TITLE OF THE INVENTION
RHESUS HER2/NEU, NUCLEOTIDES ENCODING SAME, AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Serial No. 60/437,846, filed January 3, 2003, which is hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates generally to the detection and therapy of cancer. More specifically, the present invention relates to the rhesus monkey homologue of the tumor associated polypeptide HER2/neu, to isolated nucleic acid molecules which encode this protein, and to recombinant vectors and hosts comprising DNA encoding this protein. This invention also relates to adenoviral vector constructs carrying rhesus HER2/neu and to their use in vaccines and pharmaceutical compositions for preventing and treating cancer.

BACKGROUND OF THE INVENTION

Cancer typically involves the deregulation of genes that contribute to maintaining the cell cycle or controlling cell proliferation, such as growth factors and their receptors, oncogenes and tumor suppressor genes. The products of many of these genes are expressed on the surface of a wide variety of tumor cells and, hence, were designated tumor-associated antigens (TAA). Recent evidence supports the existence of tumor-associated antigens that are capable of eliciting an immune response, making these molecules a target for vaccine therapy. Because many of these gene products are also expressed in normal cells, albeit at lower levels, many cancer vaccines targeting tumor-associated antigens have proven ineffective due to immunotolerance.

The product of the HER2/neu proto-oncogene (also called c-erbB-2) is a transmembrane TAA that is a member of the epidermal growth factor receptor family. The HER2/neu gene was originally cloned from a rat neuroglioblastoma (Shih et al., *Nature* 290: 261-264 (1981)) and later isolated and characterized from human cells (Coussens et al., *Science* 230: 1132-39 (1985); King et al., *Science* 229: 974-76 (1985)). To date, no simian homologs of HER2/neu are available.

HER2/neu has been further classified as a member of the HER family of receptor tyrosine kinases, which consists of four receptors that participate in cell growth and differentiation. The HER receptors contribute to maintaining normal cell growth by binding growth factor ligands as dimers, thereby initiating intracellular signaling cascades which

ultimately result in the activation of genes important in cell growth. Although several ligands have been identified for other members of the HER family, a high affinity ligand for the HER2/neu receptor has yet to be found (Lohrisch and Piccart, *Semin. Oncol.* 28(6): Suppl. 18: 3-11 (2001)).

Low levels of expression of the HER2/neu transcript and the encoded 185 kD protein were detected in normal adult epithelial cells of various tissues, including the skin and breast, and tissues of the gastrointestinal, reproductive and urinary tracts (Press et al., *Oncogene* 5: 953-962 (1990)). Higher levels of HER2/neu expression were also detected in the corresponding fetal tissues during embryonic development (Press et al., *supra*).

HER2/neu is commonly overexpressed or amplified in various malignancies such as carcinomas of the breast, ovary, uterus, colon, and prostate, and adenocarcinomas of the lung (reviewed in Disis and Cheever, *Adv. Cancer Research* 71: 343-371 (1997)). Such overexpression of HER2/neu correlates with a poor prognosis and a higher relapse rate for cancer patients (Slamon et al., *Science* 244: 707-712 (1989)).

Many cancer patients suffering from malignancies associated with HER2/neu overexpression have had immune responses against the protein product of the HER2/neu oncogene, thus making HER2/neu an immunological target for the development of cancer therapeutics. An effective vaccine exploiting this immune response to HER2/neu must both enhance this immunity to a level that is protective and/or preventive and overcome self-tolerance.

HER2/neu has been proposed as a target for the development of immunological treatments of different malignancies. Different anti-HER2 monoclonal antibodies have been investigated as therapies for breast cancer, with each antibody demonstrating various levels of success (for discussion, see Yarden, *Oncology* 61(suppl 2): 1-13 (2001)). Amici et al. (U.S. Patent No. 6,127,344) disclose a method for inducing immunity against HER2/neu by administering an expression vector comprising the full-length human HER2/neu cDNA functionally linked to the human cytomegalovirus promoter. Cheever and Disis disclose methods for immunizing humans against HER2/neu-associated cancers with HER2 peptides (U.S. Patent No. 5,846,538). Additionally, HER2/neu peptide-based vaccines have been studied in rodent models (for review, see Disis and Cheever, *Advances in Cancer Research* 71:343-71 (1997)).

Despite the identification of the HER2/neu clones mentioned above, it would be highly desirable to identify additional mammalian genes encoding HER2/neu to allow for the development of a cancer vaccine which is efficacious and not hindered by self-tolerance.

SUMMARY OF THE INVENTION

The present invention relates to isolated or purified nucleic acid molecules (polynucleotides) comprising a sequence of nucleotides that encode a novel rhesus monkey HER2/neu protein (also called c-erbB-2, hereinafter designated rhHER2/neu) as set forth in SEQ ID NO:2 and SEQ ID NO:41. The DNA molecules disclosed herein may be transfected into a host cell of choice wherein the recombinant host cell provides a source for substantial levels of an expressed functional rhHER2/neu protein (SEQ ID NO:2 or SEQ ID NO:41).

The present invention further relates to an isolated nucleic acid molecule which encodes mRNA that expresses a novel rhesus monkey HER2/neu protein; this DNA molecule comprising the nucleotide sequence disclosed herein as SEQ ID NO:1. A preferred aspect of this portion of the present invention is disclosed in FIGURE 1, which shows a DNA molecule (SEQ ID NO:1) that encodes a novel rhHER2/neu protein (SEQ ID NO:2).

The present invention also provides an isolated nucleic acid molecule which encodes mRNA that expresses a novel rhesus monkey HER2/neu protein; this DNA molecule comprising the nucleotide sequence disclosed herein as SEQ ID NO:40. A preferred aspect of this portion of the present invention is disclosed in FIGURE 5, which shows a DNA molecule (SEQ ID NO:40) that encodes a novel rhHER2/neu protein (SEQ ID NO:41).

The present invention also relates to recombinant vectors and recombinant host cells, both prokaryotic and eukaryotic, which contain the nucleic acid molecules disclosed throughout this specification.

The present invention further relates to a process for expressing a rhesus monkey HER2/neu protein in a recombinant host cell, comprising: (a) introducing a vector comprising the nucleic acid as set forth in SEQ ID NO:1 or SEQ ID NO:40 into a suitable host cell; and, (b) culturing the host cell under conditions which allow expression of said rhesus monkey HER2/neu protein.

A preferred aspect of the present invention is a substantially purified form of a rhesus monkey HER2/neu protein which consists of the amino acid sequence disclosed in FIGURE 2 (SEQ ID NO:2).

A preferred aspect of the present invention is a substantially purified form of a rhesus monkey HER2/neu protein which consists of the amino acid sequence disclosed in FIGURE 6 (SEQ ID NO:41).

Yet another preferred aspect of the present invention relates to a substantially purified, fully processed (including proteolytic processing, glycosylation and/or phosphorylation), mature rhHER2/neu protein obtained from a recombinant host cell containing a DNA expression vector comprising nucleotide sequence as set forth in SEQ ID NO:1 or SEQ

ID NO:40, which express the rhHER2/neu protein. It is especially preferred that the recombinant host cell be a eukaryotic host cell, such as a mammalian cell line.

Another aspect of this invention is a method of preventing or treating cancer comprising administering to a mammal a vaccine vector comprising an isolated nucleic acid molecule, the isolated nucleic acid molecule comprising a sequence of nucleotides that encodes a rhesus monkey HER2/neu protein as set forth in SEQ ID NO:2 or SEQ ID NO:41.

The present invention further relates to an adenovirus vaccine vector comprising an adenoviral genome with a deletion in the E1 and E3 regions, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising: (a) a polynucleotide encoding a rhesus monkey HER2/neu protein; and (b) a promoter operably linked to the polynucleotide.

The present invention also relates to a vaccine plasmid comprising a plasmid portion and an expression cassette portion, the expression cassette portion comprising: (a) a polynucleotide encoding a rhesus monkey HER2/neu protein; and (b) a promoter operably linked to the polynucleotide.

Another aspect of the present invention is a method of protecting or a mammal from cancer or treating a mammal suffering from cancer comprising: (a) introducing into the mammal a first vector comprising: i) a polynucleotide encoding a rhesus monkey HER2/neu protein; and ii) a promoter operably linked to the polynucleotide; (b) allowing a predetermined amount of time to pass; and (c) introducing into the mammal a second vector comprising: i) a polynucleotide encoding a rhesus monkey HER2/neu protein; and ii) a promoter operably linked to the polynucleotide.

As used throughout the specification and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

As used throughout the specification and appended claims, the following definitions and abbreviations apply:

The term "promoter" refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibiting sequences termed "silencers".

The term "cassette" refers to the sequence of the present invention that contains the nucleic acid sequence which is to be expressed. The cassette is similar in concept to a cassette tape; each cassette has its own sequence. Thus by interchanging the cassette, the vector will express a different sequence. Because of the restriction sites at the 5' and 3' ends, the cassette can be easily inserted, removed or replaced with another cassette.

The term "vector" refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, virus (including adenovirus), bacteriophages and cosmids.

The term "first generation," as used in reference to adenoviral vectors, describes said adenoviral vectors that are replication-defective. First generation adenovirus vectors typically have a deleted or inactivated E1 gene region, and preferably have a deleted or inactivated E3 gene region.

The designation "pMRKAd5-rhHER2/neu" refers to a plasmid construct, disclosed herein, which comprises an Ad5 adenoviral genome deleted of the E1 and E3 regions. In this plasmid, the E1 region is replaced by a rhesus HER2/neu gene in an E1 parallel orientation, under the control of a human CMV promoter without intron A, followed by a bovine growth hormone polyadenylation signal.

The designation "MRKAd5-rhHER2/neu" refers to the virus generated from plasmid pMRKAD5-rhHER2/neu following removal of plasmid sequences by restriction and transfection into an E1-expressing cell line, such as Per.C6 or HEK 293.

The designation "pV1J-rhHER2/neu" refers to a plasmid construct disclosed herein comprising the human CMV immediate-early (IE) promoter and intron A, a full-length rhesus HER2/neu gene, a bovine growth hormone-derived polyadenylation and transcriptional termination sequences, and a minimal pUC backbone.

The term "first rhesus HER2/neu DNA sequence," as used interchangeably with the term "rhHER2#1," refers to the rhesus HER2/neu sequence as identified and isolated herein in EXAMPLE 1 and set forth in SEQ ID NO:1. This sequence was translated to determine the amino acid sequence of the "first rhesus HER2/neu protein," as set forth in SEQ ID NO:2.

The term "second rhesus HER2/neu DNA sequence," as used interchangeably with the term "rhHER2#2," refers to the rhesus HER2/neu sequence as identified and isolated herein in EXAMPLE 4 and set forth in SEQ ID NO:40. This DNA molecule was isolated from a different rhesus monkey than the DNA molecule described in EXAMPLE 1. This sequence was translated to deduce the amino acid sequence of the "second rhesus HER2/neu protein," as set forth in SEQ ID NO:41. Differences between the rhHER2#1 and rhHER2#2 nucleotide and amino acid sequences are detailed in FIGURE 7.

The term "effective amount" means sufficient vaccine composition is introduced to produce the adequate levels of the polypeptide, so that an immune response results. One skilled in the art recognizes that this level may vary.

"Substantially free from other nucleic acids" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other nucleic acids. As used

interchangeably, the terms "substantially free from other nucleic acids," "substantially purified," "isolated nucleic acid" or "purified nucleic acid" also refer to DNA molecules which comprise a coding region for a rhesus HER2/neu protein that has been purified away from other cellular components. Thus, a rhesus HER2/neu DNA preparation that is substantially free from other nucleic acids will contain, as a percent of its total nucleic acid, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-rhesus HER2/neu nucleic acids. Whether a given rhesus HER2/neu DNA preparation is substantially free from other nucleic acids can be determined by such conventional techniques of assessing nucleic acid purity as, *e.g.*, agarose gel electrophoresis combined with appropriate staining methods, *e.g.*, ethidium bromide staining, or by sequencing.

"Substantially free from other proteins" or "substantially purified" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other proteins. Thus, a rhesus monkey HER2/neu protein preparation that is substantially free from other proteins will contain, as a percent of its total protein, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-rhesus monkey HER2/neu proteins. Whether a given rhesus monkey HER2/neu protein preparation is substantially free from other proteins can be determined by such conventional techniques of assessing protein purity as, *e.g.*, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with appropriate detection methods, *e.g.*, silver staining or immunoblotting.

As used interchangeably, the terms "substantially free from other proteins" or "substantially purified," or "isolated rhesus monkey HER2/neu protein" or "purified rhesus monkey HER2/neu protein" also refer to rhesus monkey HER2/neu protein that has been isolated from a natural source. Use of the term "isolated" or "purified" indicates that rhesus monkey HER2/neu protein has been removed from its normal cellular environment. Thus, an isolated rhesus monkey HER2/neu protein may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that an isolated rhesus monkey HER2/neu protein is the only protein present, but instead means that an isolated rhHER2/neu protein is substantially free of other proteins and non-amino acid material (*e.g.*, nucleic acids, lipids, carbohydrates) naturally associated with the rhHER2/neu protein *in vivo*. Thus, a rhesus monkey HER2/neu protein that is recombinantly expressed in a prokaryotic or eukaryotic cell and substantially purified from this host cell which does not naturally (*i.e.*, without intervention) express this rhHER2/neu protein is of course "isolated rhesus monkey HER2/neu protein" under any circumstances referred to herein. As noted above, a rhHER2/neu protein preparation that is an isolated or purified rhHER2/neu protein will be

substantially free from other proteins and will contain, as a percent of its total protein, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-rhesus monkey HER2/neu proteins.

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid for aspartic acid).

The term "mammalian" refers to any mammal, including a human being.

The abbreviation "Ag" refers to an antigen.

The abbreviations "Ab" and "mAb" refer to an antibody and a monoclonal antibody, respectively.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the nucleotide sequence of the first rhesus monkey HER2/neu cDNA, as set forth in SEQ ID NO:1 (see EXAMPLE 1). The presence of an "R" at position 795 indicates that either an A or a G is located at that position.

FIGURE 2 shows the predicted amino acid sequence of rhesus monkey HER2/neu protein, as set forth in SEQ ID NO:2. The amino acid sequence shown was deduced from the nucleotide sequence disclosed as SEQ ID NO:1.

FIGURE 3 discloses the nucleotide sequences of oligonucleotide primers spanning the HER2/neu gene, which were used to generate a series of rhesus HER2/neu fragments by RT-PCR (see EXAMPLE 1). Columns marked "Forward" and "Reverse" state the location of the primers with respect to the published human HER2/neu sequence (Accession M11730). Primers disclosed as SEQ ID NOs: 15 and 16 were designed to mutate amino acid position 579 from K to A, to inactivate the tyrosine kinase activity of the translated protein. Primers 3388-3410 and 3410-3388 have a sequence with a C at position 15 and a G at position 10, respectively, which code for Ser (AGC). In contrast, the Rhesus HER2/neu sequence has a T at that position, coding again for Ser (AGT). These primers were used for sequencing, but not for cloning purposes. For SEQ ID NOs: 11 and 19, sequence priming on rhHER2/neu is underlined.

FIGURE 4 shows the sequences of RT-PCR primers used to construct the full-length rhesus HER2/neu clone (see EXAMPLE 2). The first column lists the reaction number corresponding to the reactions discussed in EXAMPLE 2. Each row depicts a set of forward and reverse primers used to generate the clones listed in column 4. Nomenclature for the clones

indicates both the vector used for cloning (BS or CR for pBluescript or pCRII, respectively) and the location of the sequence relative to the published human HER2/neu sequence (listed as numbers).

FIGURE 5 shows the nucleotide sequence of the second rhesus monkey HER2/neu cDNA, as set forth in SEQ ID NO:40 (see EXAMPLE 4). The presence of an "R" within the nucleotide sequence indicates that either an "A" or a "G" is located at that position. The presence of a "Y" within the sequence indicates that a "C" or a "T" is located at that position. Nucleotide bases that are different from the corresponding bases of the first rhesus HER2/neu (SEQ ID NO:1) are bold and underlined.

FIGURE 6 shows the predicted amino acid sequence of the second rhesus monkey HER2/neu protein, as set forth in SEQ ID NO:41 (see EXAMPLE 4). The amino acid sequence shown was deduced from the nucleotide sequence disclosed as SEQ ID NO:40. The "X" at position 517 indicates that a "Q" (Gln) or an "R" (Arg) may be present at that position. The "X" at position 647 indicates that a "K" (Lys) or an "R" (Arg) may be present at that position. The "X" at position 1075 indicates that an "R" (Arg) or a "Q" (Gln) may be present at that position.

FIGURE 7 details the specific mutations present in the second rhesus HER2/neu DNA and protein sequences (RhHER2#2, SEQ ID NOs:40 and 41) as compared to the first rhesus HER2/neu DNA and protein sequences (RhHER2#1, SEQ ID NOs: 1 and 2). The first column in each table lists the position of nucleotides that are different between RhHER2#2 and RhHER2#1. The second column in each table list the number of specific clones carrying HER2/neu fragments that were isolated and used to determine the sequence of RhHER2#1 and RhHER2#2, respectively. The third column in each table shows the sequence of the codon in which the differences occur, with dissimilar nucleotides highlighted. Below the codons are the one-letter amino acid symbols for the resulting amino acids, highlighted in gray.

DETAILED DESCRIPTION OF THE INVENTION

The gene encoding the HER2/neu tumor-associated antigen is commonly associated with the development of epithelial-derived human carcinomas. The present invention relates to compositions and methods to elicit or enhance immunity to the protein product expressed by the HER2/neu tumor-associated antigen, wherein aberrant HER2/neu expression is associated with the carcinoma or its development. Association of aberrant HER2/neu expression with a carcinoma does not require that the HER2/neu protein be expressed in tumor tissue at all timepoints of its development, as abnormal HER2/neu expression may be present at tumor initiation and not be detectable late into tumor progression.

To this end, polynucleotides encoding rhesus monkey HER2/neu are provided. The molecules of the present invention may be used in a recombinant adenovirus vaccine to provide effective immunoprophylaxis against epithelial-derived carcinomas through cell-mediated immunity. When directly introduced into a vertebrate *in vivo,* the invention polynucleotides induce the expression of encoded proteins within the animal, including mammals such as primates, dogs and humans.

The present invention relates to an isolated nucleic acid molecule (polynucleotide) comprising a sequence of nucleotides which encodes mRNA that expresses a novel rhHER2/neu protein as set forth in SEQ ID NO:2. The present invention also relates to an isolated nucleic acid molecule comprising a sequence of nucleotides which encodes mRNA that expresses a novel rhHER2/neu protein as set forth in SEQ ID NO:41. The nucleic acid molecules of the present invention are substantially free from other nucleic acids.

The isolated nucleic acid molecules of the present invention may include a deoxyribonucleic acid molecule (DNA), such as genomic DNA and complementary DNA (cDNA), which may be single (coding or noncoding strand) or double stranded, as well as synthetic DNA, such as a synthesized, single stranded polynucleotide. The isolated nucleic acid molecules of the present invention may also include a ribonucleic acid molecule (RNA). For most cloning purposes, DNA is a preferred nucleic acid.

A preferred DNA molecule of the present invention comprises the nucleotide sequence disclosed herein as SEQ ID NO:1, shown in FIGURE 1, which encodes the rhesus HER2/neu protein shown in FIGURE 2 and set forth as SEQ ID NO:2. This rhHER2/neu nucleic acid molecule was identified through RT-PCR as described in detail in EXAMPLE 1. The presence of an "R" at position 795 of SEQ ID NO:1 indicates that clones isolated from the first rhesus monkey comprised either an A or a G at that position. A nucleic acid molecule isolated from the first rhesus monkey and comprising an "A" at position 795 is designated herein SEQ ID NO:42. A nucleic acid molecule isolated from the first rhesus monkey and comprising a "G" at position 795 is designated herein SEQ ID NO:43.

A second preferred DNA molecule comprises the nucleotide sequence disclosed herein as SEQ ID NO:40, shown in FIGURE 5, which encodes the rhesus HER2/neu protein shown in FIGURE 6 and set forth as SEQ ID NO:41. The isolated cDNA clones, associated vectors, hosts, recombinant subcellular fractions and membranes, and the expressed and mature forms of rhHER2/neu are useful for the development of a cancer vaccine.

The present invention also includes biologically active fragments or mutants of SEQ ID NO:1 or SEQ ID NO:40, which encode mRNA expressing novel rhHER2/neu proteins. Any such biologically active fragment and/or mutant will encode either a protein or protein

fragment which at least substantially mimics the pharmacological properties of the rhHER2/neu protein, including but not limited to the rhHER2/neu proteins as set forth in SEQ ID NO:2 and SEQ ID NO:41. Any such polynucleotide includes but is not necessarily limited to: nucleotide substitutions, deletions, additions, amino-terminal truncations and carboxy-terminal truncations. The mutations of the present invention encode mRNA molecules that express a functional rhHER2/neu protein in a eukaryotic cell so as to be useful in cancer vaccine development.

This invention also relates to synthetic DNA that encodes the rhHER2/neu protein where the nucleotide sequence of the synthetic DNA differs significantly from the nucleotide sequence of SEQ ID NO:1 and SEQ ID NO:40, but still encodes the same rhHER2/neu protein as SEQ ID NO:2 or SEQ ID NO:41. Such synthetic DNAs are intended to be within the scope of the present invention.

Therefore, the present invention discloses codon redundancy that may result in numerous DNA molecules expressing an identical protein. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein that do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in the functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide that has properties that are different than those of the naturally occurring peptide. Methods of altering the DNA sequences include but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or receptor for a ligand.

Included in the present invention are DNA sequences that hybridize to SEQ ID NO:1 or SEQ ID NO:40 under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hours to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography. Other procedures using conditions of high stringency would include either a hybridization step carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 which is hereby incorporated by reference. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

A preferred aspect of the present invention is a substantially purified form of a rhesus monkey HER2/neu protein which comprises a sequence of amino acids as disclosed in FIGURE 2 (SEQ ID NO:2).

Another preferred aspect of the present invention is a substantially purified form of a rhesus monkey HER2/neu protein which comprises a sequence of amino acids as disclosed in FIGURE 6 (SEQ ID NO:41).

This invention also relates to various functional domains of rhHER2/neu, such as the extracellular domain and the intracellular domain, and to hybrid molecules comprising at least one of these sequences.

The present invention also includes biologically active fragments and/or mutants of a rhHER2/neu protein, comprising the amino acid sequence as set forth in SEQ ID NO: 2 or SEQ ID NO:41, including but not necessarily limited to amino acid substitutions, deletions, additions, amino terminal truncations and carboxy-terminal truncations such that these mutations provide for proteins or protein fragments of diagnostic, therapeutic or prophylactic use and would be useful for cancer vaccine development.

The rhesus monkey HER2/neu proteins of the present invention may be in the form of the "mature" protein or may be a part of a larger protein such as a fusion protein. It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification such as multiple histidine residues, or an additional sequence for stability during recombinant production.

The present invention also relates to rhHER2/neu fusion constructs, including but not limited to fusion constructs which express a portion of the rhesus HER2/neu protein linked to various markers, including but in no way limited to GFP (Green fluorescent protein), the MYC epitope, GST, and Fc. Any such fusion construct may be expressed in the cell line of interest and used to screen for modulators of the rhesus HER2/neu protein disclosed herein.

The present invention further relates to recombinant vectors that comprise the substantially purified nucleic acid molecules disclosed throughout this specification. These vectors may be comprised of DNA or RNA. For most cloning purposes, DNA vectors are preferred. Typical vectors include plasmids, modified viruses, bacteriophage, cosmids, yeast

artificial chromosomes, and other forms of episomal or integrated DNA that can encode a rhHER2/neu protein. It is well within the purview of the skilled artisan to determine an appropriate vector for a particular gene transfer or other use.

An expression vector containing DNA encoding a rhHER2/neu protein may be used for expression of rhHER2/neu in a recombinant host cell. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Also, a variety of bacterial expression vectors may be used to express recombinant rhHER2/neu in bacterial cells if desired. In addition, a variety of fungal cell expression vectors may be used to express recombinant rhHER2/neu in fungal cells. Further, a variety of insect cell expression vectors may be used to express recombinant protein in insect cells.

The present invention also relates to host cells transformed or transfected with vectors comprising the nucleic acid molecules of the present invention. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of bovine, porcine, monkey and rodent origin; and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Such recombinant host cells can be cultured under suitable conditions to produce rhHER2/neu or a biologically equivalent form.

As noted above, an expression vector containing DNA encoding a rhHER2/neu protein may be used for expression of rhHER2/neu in a recombinant host cell. Therefore, another aspect of this invention is a process for expressing a rhesus monkey HER2/neu protein in a recombinant host cell, comprising: (a) introducing a vector comprising the nucleic acid of as set forth in SEQ ID NO:1 or SEQ ID NO:40 into a suitable host cell; and, (b) culturing the host cell under conditions which allow expression of said rhesus monkey HER2/neu protein.

Following expression of rhHER2/neu in a host cell, rhHER2/neu protein may be recovered to provide rhHER2/neu protein in active form. Several rhHER2/neu protein purification procedures are available and suitable for use. Recombinant rhHER2/neu protein may be purified from cell lysates and extracts by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography. In addition, recombinant rhHER2/neu protein can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for full-length rhHER2/neu protein, or polypeptide fragments of rhHER2/neu protein.

The nucleic acids of the present invention may be assembled into an expression cassette that comprises sequences designed to provide for efficient expression of the protein in a human cell. The cassette preferably contains the full-length rhHER2/neu gene, with related

transcriptional and translations control sequences operatively linked to it, such as a promoter, and termination sequences. In a preferred embodiment, the promoter is the cytomegalovirus promoter without the intron A sequence, although those skilled in the art will recognize that any of a number of other known promoters such as the strong immunoglobulin, or other eukaryotic gene promoters may be used. A preferred transcriptional terminator is the bovine growth hormone terminator, although other known transcriptional terminators may also be used. The combination of CMV-BGH terminator is particularly preferred.

In accordance with this invention, the rhesus HER2/neu expression cassette is inserted into a vector. The vector is preferably an adenoviral vector, although linear DNA linked to a promoter, or other vectors, such as adeno-associated virus or a modified vaccinia virus vector may also be used.

If the vector chosen is an adenovirus, it is preferred that the vector be a so-called first-generation adenoviral vector. These adenoviral vectors are characterized by having a non-functional E1 gene region, and preferably a deleted adenoviral E1 gene region. In some embodiments, the expression cassette is inserted in the position where the adenoviral E1 gene is normally located. In addition, these vectors optionally have a non-functional or deleted E3 region. It is preferred that the adenovirus genome used be deleted of both the E1 and E3 regions (ΔE1ΔE3). The adenoviruses can be multiplied in known cell lines which express the viral E1 gene, such as 293 cells, or PERC.6 cells, or in cell lines derived from 293 or PERC.6 cell which are transiently or stablily transformed to express an extra protein. For examples, when using constructs that have a controlled gene expression, such as a tetracycline regulatable promoter system, the cell line may express components involved in the regulatory system. One example of such a cell line is T-Rex-293; others are known in the art.

For convenience in manipulating the adenoviral vector, the adenovirus may be in a shuttle plasmid form. This invention is also directed to a shuttle plasmid vector which comprises a plasmid portion and an adenovirus portion, the adenovirus portion comprising an adenoviral genome which has a deleted E1 and optional E3 deletion, and has an inserted expression cassette comprising rhesus HER2/neu. In preferred embodiments, there is a restriction site flanking the adenoviral portion of the plasmid so that the adenoviral vector can easily be removed. The shuttle plasmid may be replicated in prokaryotic cells or eukaryotic cells.

In a preferred embodiment of the invention, the expression cassette is inserted into the pMRKAd5-HV0 adenovirus plasmid (*See* Emini et al., WO 02/22080, which is hereby incorporated by reference). This plasmid comprises an Ad5 adenoviral genome deleted of the E1 and E3 regions. The design of the pMRKAd5-HV0 plasmid was improved over prior

adenovectors by extending the 5' cis-acting packaging region further into the E1 gene to incorporate elements found to be important in optimizing viral packaging, resulting in enhanced virus amplification. Advantageously, this enhanced adenoviral vector is capable of maintaining genetic stability following high passage propagation.

Standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the adenoviruses, shuttle plasmids, and DNA immunogens of this invention.

The vectors described above may be used in immunogenic compositions and vaccines for preventing the development of epithelial-derived carcinomas associated with aberrant HER2/neu expression and/or for treating existing cancers. To this end, one aspect of the instant invention is a method of preventing or treating cancer comprising administering to a mammal a vaccine vector comprising an isolated nucleic acid molecule, the isolated nucleic acid molecule comprising a sequence of nucleotides that encodes a rhesus monkey HER2/neu protein as set forth in SEQ ID NO:2 or SEQ ID NO:41.

In accordance with the method described above, the vaccine vector may be administered for the treatment or prevention of cancer in any mammal. In a preferred embodiment of the invention, the mammal is a human.

Further, one of skill in the art may choose any type of vector for use in the treatment and prevention method described. Preferably, the vector is an adenovirus vector or a plasmid vector. In a preferred embodiment of the invention, the vector is an adenoviral vector comprising an adenoviral genome with a deletion in the adenovirus E1 region, and an insert in the adenovirus E1 region, wherein the insert comprises an expression cassette comprising: (a) a polynucleotide encoding a rhesus monkey HER2/neu protein; and (b) a promoter operably linked to the polynucleotide.

The instant invention further relates to an adenovirus vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising: (a) a polynucleotide encoding a rhesus monkey HER2/neu protein; and (b) a promoter operably linked to the polynucleotide.

In a preferred embodiment of this aspect of the invention, the adenovirus vector is an Ad 5 vector.

In another aspect, the invention relates to a vaccine plasmid comprising a plasmid portion and an expression cassette portion, the expression cassette portion comprising: (a) a polynucleotide encoding a rhesus monkey HER2/neu protein; and (b) a promoter operably linked to the polynucleotide.

In some embodiments of this invention, the recombinant adenovirus vaccines disclosed herein are used in various prime/boost combinations with a plasmid-based polynucleotide vaccine in order to induce an enhanced immune response. In this case, the two vectors are administered in a "prime and boost" regimen. For example the first type of vector is administered, then after a predetermined amount of time, for example, 1 month, 2 months, six months, or other appropriate interval, a second type of vector is administered. Preferably the vectors carry expression cassettes encoding the same polynucleotide or combination of polynucleotides. In the embodiment where a plasmid DNA is also used, it is preferred that the vector contain one or more promoters recognized by mammalian or insect cells. In a preferred embodiment, the plasmid would contain a strong promoter such as, but not limited to, the human CMV promoter. The rhesus HER2/neu gene or other gene to be expressed would be linked to such a promoter. An example of such a plasmid would be the mammalian expression plasmid V1Jns as described (J. Shiver et. al. in *DNA Vaccines*, M. Liu et al. eds., N.Y. Acad. Sci., N.Y., 772:198-208 (1996), which is herein incorporated by reference).

As stated above, an adenoviral vector vaccine and a plasmid vaccine may be administered to a vertebrate as part of a single therapeutic regime to induce an immune response. To this end, the present invention relates to a method of protecting a mammal from cancer comprising: (a) introducing into the mammal a first vector comprising: i) a polynucleotide encoding a rhesus monkey HER2/neu protein; and ii) a promoter operably linked to the polynucleotide; (b) allowing a predetermined amount of time to pass; and (c) introducing into the mammal a second vector comprising: i) a polynucleotide encoding a rhesus monkey HER2/neu protein; and ii) a promoter operably linked to the polynucleotide.

In one embodiment of the method of protection described above, the first vector is a plasmid and the second vector is an adenovirus vector. In an alternative embodiment, the first vector is an adenovirus vector and the second vector is a plasmid.

The instant invention further relates to a method of treating a mammal suffering from an epithelial-derived carcinoma comprising: (a) introducing into the mammal a first vector comprising: i) a polynucleotide encoding a rhesus monkey HER2/neu protein; and ii) a promoter operably linked to the polynucleotide; (b) allowing a predetermined amount of time to pass; and (c) introducing into the mammal a second vector comprising: i) a polynucleotide encoding a rhesus monkey HER2/neu protein; and ii) a promoter operably linked to the polynucleotide.

In one embodiment of the method of treatment described above, the first vector is a plasmid and the second vector is an adenovirus vector. In an alternative embodiment, the first vector is an adenovirus vector and the second vector is a plasmid.

The amount of expressible DNA or transcribed RNA to be introduced into a vaccine recipient will depend partially on the strength of the promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 ng to 100 mg, and preferably about 10μg to 300 μg of a plasmid vaccine vector is administered directly into muscle tissue. An effective dose for recombinant adenovirus is approximately $10^6 - 10^{12}$ particles and preferably about 10^7—10^{11}particles. Subcutaneous injection, intradermal introduction, impression though the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations may be provided. Parentaeral administration, such as intravenous, intramuscular, subcutaneous or other means of administration with adjuvants such as interleukin 12 protein, concurrently with or subsequent to parenteral introduction of the vaccine of this invention is also advantageous.

The vaccine vectors of this invention may be naked, i.e., unassociated with any proteins, adjuvants or other agents which impact on the recipient's immune system. In this case, it is desirable for the vaccine vectors to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline . Alternatively, it may be advantageous to administer an immunostimulant, such as an adjuvant, cytokine, protein, or other carrier with the vaccines or immunogenic compositions of the present invention. Therefore, this invention includes the use of such immunostimulants in conjunction with the compositions and methods of the present invention. An immunostimulant, as used herein, refers to essentially any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an exogenous antigen. Said immunustimulants can be administered in the form of DNA or protein. Any of a variety of immunostimulants may be employed in conjunction with the vaccines and immunogenic compositions of the present inventions, including, but not limited to: GM-CSF, IFNα, tetanus toxoid, IL12, B7.1, LFA-3 and ICAM-1. Said immunostimulants are well-known in the art. Agents which assist in the cellular uptake of DNA, such as, but not limited to calcium ion, may also be used. These agents are generally referred to as transfection facilitating reagents and pharmaceutically acceptable carriers. Those of skill in the art will be able to determine the particular immunostimulant or pharmaceutically acceptable carrier as well as the appropriate time and mode of administration.

Any of a variety of procedures may be used to clone rhHER2/neu. These methods include, but are not limited to, (1) a RACE PCR cloning technique (Frohman et al., *Proc. Natl. Acad. Sci. USA* 85: 8998-9002 (1988)). 5′ and/or 3′ RACE may be performed to generate a full-length cDNA sequence. This strategy involves using gene-specific oligonucleotide primers for PCR amplification of rhHER2/neu cDNA. These gene-specific

primers are designed through identification of an expressed sequence tag (EST) nucleotide sequence which has been identified by searching any number of publicly available nucleic acid and protein databases; (2) direct functional expression of the rhHER2/neu cDNA following the construction of a rhHER2/neu-containing cDNA library in an appropriate expression vector system; (3) screening an rhHER2/neu-containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a labeled degenerate oligonucleotide probe designed from the amino acid sequence of the rHER2/neu protein; (4) screening an rhHER2/neu-containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a partial cDNA encoding the rhHER2/neu protein. This partial cDNA is obtained by the specific PCR amplification of rhHER2/neu DNA fragments through the design of degenerate oligonucleotide primers from the amino acid sequence known for other growth factor receptors which are related to the rhHER2/neu protein; (5) screening a rhHER2/neu-containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a partial cDNA or oligonucleotide with homology to a mammalian rhHER2/neu protein. This strategy may also involve using gene-specific oligonucleotide primers for PCR amplification of rhHER2/neu cDNA identified as an EST as described above; or (6) designing 5′ and 3′ gene specific oligonucleotides using SEQ ID NO: 1 or SEQ ID NO:40 as a template so that either the full-length cDNA may be generated by known RACE techniques, or a portion of the coding region may be generated by these same known RACE techniques to generate and isolate a portion of the coding region to use as a probe to screen one of numerous types of cDNA and/or genomic libraries in order to isolate a full-length version of the nucleotide sequence encoding rhHER2/neu.

It is readily apparent to those skilled in the art that other types of libraries, as well as libraries constructed from other cell types-or species types, may be useful for isolating a rhHER2/neu-encoding DNA or a rhHER2/neu homologue. Other types of libraries include, but are not limited to, cDNA libraries derived from other cells.

It is also readily apparent to those skilled in the art that suitable cDNA libraries may be prepared from cells or cell lines which have rhHER2/neu activity, such as various epithelial-derived cells. The selection of cells or cell lines for use in preparing a cDNA library to isolate a cDNA encoding rhHER2/neu may be done by first measuring cell-associated rhHER2/neu activity using any known assay available for such a purpose.

Preparation of cDNA libraries can be performed by standard techniques well known in the art. Well known cDNA library construction techniques can be found for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989. Complementary DNA libraries may also be obtained

from numerous commercial sources, including but not limited to Clontech Laboratories, Inc. (Palo Alto, CA) and Stratagene (La Jolla, CA).

The DNA molecules, RNA molecules, and recombinant protein of the present invention may be used to screen and measure levels of rhHER2/neu. The recombinant proteins, DNA molecules, and RNA molecules lend themselves to the formulation of kits suitable for the detection and typing of rhHER2/neu. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant rhHER2/neu or anti-rhHER2/neu antibodies suitable for detecting rhHER2/neu. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing methodologies and materials that might be used in connection with the present invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Having described preferred embodiments of the invention with reference to the accompanying drawings, it is to be understood that the invention is not limited to those precise embodiments, and that various changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims.

The following examples illustrate, but do not limit the invention.

EXAMPLE 1

Isolation of the Rhesus HER2/neu cDNA by RT-PCR

Molecular procedures were performed following standard procedures well known in the art (*See; e.g.*, Ausubel et. al. *Short Protocols in Molecular Biology*, F.M., -2nd. ed., John Wiley & Sons, (1992) and Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press (1989), which are hereby incorporated by reference).

HER2/neu nucleotide sequences from human, hamster, dog, and rat were aligned to identify highly conserved regions of the HER2/neu DNA. Based on the sequence comparison, oligonucleotide primers spanning the HER2/neu gene were designed for amplification of the rhesus HER2/neu cDNA by reverse transcriptase polymerase chain reaction (RT-PCR), described below. (see FIGURE 3).

Colon biopsies from two different Rhesus monkeys (*macaca mulatta*) were obtained from Dr. Willem Collignon (Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands). RNA was extracted and purified from each colon biopsy using the UltraSPec-

II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer's instructions. To isolate the rhesus HER2/neu gene, RT-PCR amplification products covering the entire HER2/neu sequence were generated from total RNA isolated from a single rhesus monkey.

To perform the reverse transcription step, total RNA samples were reverse transcribed using the Superscript One-Step RT-PCR Amplification Kit for Long Templates (Life Technologies; Carlsbad, CA) according to the manufacturer's instructions. Typically, 0.5-2.0 μg RNA were combined with the reverse transcriptase enzyme and the appropriate buffer in a 50μl reaction volume. Samples were incubated at 45 °C for 30 min, followed by a 2 minute incubation at 94 °C.

Using the resulting cDNA templates, PCR amplifications were performed using different combinations of forward and reverse primers (see FIGURE 3). PCR was carried out in a Perkin Elmer 2400 thermocycler (Perkin Elmer, Inc., Wellesley, MA). Cycling conditions consisted of 35 cycles of an initial denaturation step of 94°C for 15 sec, followed by a primer annealing step and concluding with an extension step. The primer annealing step consisted of incubation for 50 sec at a temperature ranging from (50°C -51°C), depending on the primer sequence. The extension step consisted of an incubation at 68°C for a length of time ranging from (80 sec-100 sec), depending on the expected length of the amplification product. The above 35 cycles were followed by an extensive elongation step of 7 min at 72°C.

Amplification products were gel-purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced with the same primers used for amplification. Sequencing reactions were carried out through Big Dye Terminator chemistry, using the Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Readings were performed using an ABI Prism 377 DNA sequencer (Applied Biosystem).

Data acquired by sequencing the different amplification products, which encompass the entire HER2/neu coding region, identified the rhesus HER2/neu sequence, disclosed herein as SEQ ID NO:1 (hereinafter "first rhesus HER2 nucleotide sequence" or "rhHER2#1," see FIGURE 1). The presence of an "R" at position 795 of SEQ ID NO:1 indicates that clones isolated from the first rhesus monkey comprised either an A or a G at that position. A nucleic acid molecule isolated from the first rhesus monkey and comprising an "A" at position 795 is designated herein SEQ ID NO:42. A nucleic acid molecule isolated from the first rhesus monkey and comprising a "G" at position 795 is designated herein SEQ ID NO:43. The single nucleotide change at position 795 of SEQ ID NO:1, based on DNA isolated from the first rhesus monkey, did not affect the resulting amino acid sequence of the HER2/neu protein, which is disclosed herein as SEQ ID NO:2 (see FIGURE 2).

Amplification products derived from the above RT-PCR reactions were cloned into either vector BlueScript ks(+) or pCRII, referred to herein as BS or CR, respectively. The resulting clones were sequenced to confirm the rhHER2/neu nucleotide sequence obtained by direct sequencing of amplification products and used to assemble the complete rhesus HER2/neu cDNA sequence.

EXAMPLE 2

Assembly of the Complete HER2/neu cDNA Sequence

A series of clones obtained from the first rhesus monkey were constructed and assembled by PCR to generate the complete rhesus HER2/neu cDNA. First, four RT-PCR amplification products spanning the 5' end of the rhesus HER2/neu gene were generated as described in EXAMPLE 1. The PCR - amplified fragments from the above clones were cloned into either BS or CR, as indicated in FIGURE 4 (Reactions 1-4). Sequence analysis of several clones confirmed that the cloned sequences were the same as sequences obtained from the RT-PCR fragments.

The overlapping amplication products described above were ligated in a 100µl PCR reaction in which the following components were combined: 0.1-1.0 pmol of each of the above fragments, Pfu polymerase (Stratagene, La Jolla, CA) and the appropriate buffer. Samples were subjected to an initial amplification cycle consisting of 30 sec at 95°C, followed by 4 min at 72°C.

The resulting ligation product was amplified again with the PmeI_SwaI_RBS_1-16 (SEQ ID NO:11) and 1895-1876 (SEQ ID NO:10) primers using Pfu polymerase and the appropriate buffer with the following thermal profile: 95°C for 30 sec, 58°C for 30 sec and 72°C for 180 sec (30 cycles). The PCR product thus obtained was gel purified and cloned into pCRII vector to generate clone #1_CR_1-1895.7(-) (in the anti-clockwise orientation). Sequence analysis of several clones confirmed the identity of the cloned sequence.

Colon RNA from the first rhesus monkey was used to generate an additional PCR fragment by RT-PCR, essentially as described above. Oligonucleotide primers used for this purpose were as follows: 1558-1583 (SEQ ID NO:12) and 2798-2776 (SEQ ID NO:13) (Reaction 6, FIGURE 4). This fragment, located in the center portion of the HER2/neu gene, was used as a template for further amplification, without cloning.

Primers were then designed to PCR-amplify this region from bp 1621 to bp 2277 (FIGURE 4, Reactions 7, SEQ ID NOs: 14, 15) and from bp 2239 to 2798 (FIGURE 4, Reaction 8, SEQ ID NOs: 16 and 13). The resulting products were cloned into the pCRII vector. In order to inactivate the tyrosine kinase activity of the protein, primers were designed to mutate amino

acid position 579 from K to A (SEQ ID NOs:15 and 16). Sequence analysis of several clones confirmed the identity of the cloned sequences.

Colon RNA from the first rhesus monkey was used to generate an additional PCR fragment by RT-PCR, essentially as described above. Oligonucleotide primers used for this purpose were as follows:2356-2378 (SEQ ID NO:17) and 4166-4145 (SEQ ID NO:18) (FIGURE 4, Reaction 9). The resulting product was cloned into the pCRII vector. Sequence analysis confirmed the identity of the cloned sequences.

DNA fragments were PCR amplified from the three above clones using the following primers: 1621-1644 (SEQ ID NO 14) and 2277-K753-2248 (SEQ ID NO 15) with template #1_CR_1621-2277.2; 2239-K756-2248 (SEQ ID NO 16) and 2798-2776 (SEQ ID NO 13) with template #1_CR_2239-2798.4; 2356-2378 (SEQ ID NO 17) and SalI_3768-3746 (SEQ ID NO 19) with template #1_CR_2356-4169.2. The three products were assembled by PCR as described above. The resulting ligation product was amplified using the following primers: 1621-1644 (SEQ ID NO:14) and SalI_3768-3746 '(SEQ ID NO:19) and cloned into pCRII vector in the clockwise orientation, generating clones #1-CR_1621-3768.8 (hereinafter clone 10.8) and #1-CR_1621-3768.12 (hereinafter clone 10.12) (FIGURE 4, Reactions 10). Sequencing revealed that clone 10.12 had a mutation around position 3686 in the 3' region. Simialry, clone 10.8 had a mutation around postion 2666. A wild type sequence was generated by replacinga 508 bp *BstE*II-*Sal*I fragment from clone 10.12 with the corresponding region from clone 10.8, which did not include mutations in this region. The resulting clone was named #1-CR_1621-3768.128.

A *Pml*I-*Xba*I fragment from clone #1_CR_1621_3768.128(+) was cloned into the *Pml*I-*Spe*I sites of clone #1_CR_1_1895.7(-). The resulting plasmid, which contains the entire rhesus HER2/neu coding sequence, was named #1_CR_1-3768(-). Sequence analysis confirmed the identity of the sequence.

EXAMPLE 3

Immunogens

For gene transduction and immunization studies, the rhHER2/neu coding region was excised from #1_CR_1-3768(-) by digestion with *Pme*I and *Sal*I and inserted into the *Eco*RV and *Sal*I sites of mammalian expression plasmid pV1J_nsA (Montgomery et al., *DNA Cell Biol.* 12(9): 777-83 (1993)), generating pV1J-rh-HER2/neu.

For adenovirus vector construction, the rhHER2/neu-encoding sequence was excised from #1_CR_1-3768(-) by digestion with *Pme*I and *Sal*I and cloned into the corresponding site of the ppolyMRKAd5ΔE1 shuttle plasmid, generating pMRKAd5ΔE1-

rhHER2/neu. Shuttle vector pMRKAd5ΔE1 contains Ad5 sequences from b.p. 1 to b.p. 450 and from b.p. 3511 to b.p. 5798 with an expression cassette containing human cytomegalovirus (HCMV) promoter (without intron A) and bovine growth hormone polyadenylation signal. The plasmid was recombined with the adenoviral backbone vector pMRKAd5HVO , which contains all Ad5 sequences except those encompassing the E1 and E3 regions, using *E. coli* BJ5183 cells (Chartier et al., *J. Virol.* 70: 4805-10 (1996)).

For vector production, pMRKAd5-rhHER2/neu was linearized by digestion with *Pac*I and transfected in PerC.6 cells using Lipofectamine (Life Technologies, Rockville, MD). 5-6 viral passages were performed to amplify viral titer and a large viral amplification was carried out with a final production of $1.3x10^{12}$ physical particles (pp). No genome rearrangements were detectable in the viral genome purified from the amplified vector, as indicated by restriction fragment length polymorphism (RFLP) analysis. The expected DNA fragments were observed both in the viral genome and in the control pMRKAd5-HER2/neu plasmid, restricted in parallel.

EXAMPLE 4

Isolation of HER2/neu-encoding DNA from a Second Rhesus Monkey

A similar sequencing analysis of RT-PCR products was performed using colon RNA from a second rhesus monkey (see EXAMPLE 1). Data acquired by sequencing many different amplification products encompassing the whole gene identified a second rhesus HER2/neu sequence, disclosed herein as SEQ ID NO:40, (hereinafter "second rhesus HER2 nucleotide sequence" or "rhHER2#2," see FIGURE 5), with the deduced amino acid sequence disclosed herein as SEQ ID NO:41 (hereinafter "second rhesus HER2 amino acid sequence," see FIGURE 6).

As in EXAMPLE 1, amplification products were generated by RT-PCR of total colon RNA from the second rhesus monkey using primers spanning the HER2/neu gene (see FIGURE 3). The resulting amplification products were gel-purified and sequenced. Additionally, these products were cloned into either the BS or the CR vector. DNA sequencing analysis of the resulting clones confirmed the rhHER2/neu nucleotide sequences obtained by direct sequencing of amplification products.

Eight differences were detected between rhHER2#1 and rhHER2#2 (for details, see FIGURE 7). Five of these mutations introduce an amino acid change in the protein as compared to the first rhesus HER2 amino acid sequence (SEQ ID NO:2). Of note, three of these mutations do not produce amino acid changes in the rhHER2#2 protein as compared with the rhHER2#1 protein.

EXAMPLE 5

Immunization of Rhesus Macaques with rhHER2/neu

In order to assess the efficiency of immunization of Rhesus macaques (*Macaca mulatta*) with the rhesus homologues of the human tumor antigen HER2/neu, which is expressed in colorectal carcinomas, immunization studies were performed at the Biomedical Primate Research Centre (BPRC), Rijswijk (The Netherlands). The studies were designed to evaluate both B and T cell responses to immunization with the rhesus HER2/neu antigen.

In a first study, one group of monkeys (4 rhesus monkeys total; 2 males and 2 females) were immunized with a plasmid DNA vector or an adenovirus vector expressing the rhesus homologue of the human tumor antigen HER2/neu (pV1J-rhHER2). For priming, animals were vaccinated intramuscularly (i.m.) with plasmid DNA at weeks 0, 4, 8, 12, and 16, by injection of DNA followed by electrical stimulation. The DNA was injected as a 1 ml solution (split over 2 sites with 0.5 ml/site) containing 5 mg pV1J-rhHER2 plasmid DNA for animals weighing 2-5 kilos. Animals are injected under anesthesia (mixture of ketamine/xylazine).

For electrostimulation, 2 trains of 100 square bipolar pulses (1 sec each), were delivered every other second for a total treatment time of 3 sec. The pulse length was 2 msec/phase with a pulse frequency and amplitude of 100 Hz and 100 mA (constant current mode), respectively.

The same group of animals was boosted by i.m. injection of Adenovirus 5 (Ad5) expressing rhesus HER2/neu. A ΔE1-ΔE3, "first generation" Adenovirus (P2 level) was used. A total amount of 10 exp11 viral particles (vp) were injected at week 24 and 28.

A further boosting was carried out by i.m. injection of 10exp11 viral particles (vp) of Adenovirus 24 (Ad24) expressing rhesus HER2/neu at weeks 36 and 40. Ad24 was chosen because neutralizing antibodies induced by Ad5 injection do not interfere with Ad24 infection.

To measure the immune response to HER2/neu induced by the above immunization protocol, blood samples were collected every four weeks for a total duration of one year. The HER2-specific cell mediated immune response was measured by IFNγ ELISPOT assay. The number of IFN-γ -secreting anti-rhesus HER2 T cells was determined by ELISPOT on PBMC using pools of peptides. Three hundred and eleven peptides, each 15 amino acids long, overlapping by 11 residues and spanning the entire rhesus protein sequence, were combined into eleven pools indicated with alphabetical letters from A to K (from N- to C-terminus). The frequency of IFN-γ producing PBMC was calculated as the average value of

spots derived from duplicates at two different cell concentrations. Values were expressed as the number of spot forming colonies (SFC)/10^6 total PBMC, minus the background values determined in the absence of peptides (typically less than 10 SFC/10^6 total spleen cells). Calculated results indicate that all four monkeys showed a detectable cell-mediated response, as measured by IFN-γ ELISPOT.

Reactivity was confirmed and typed by IFN-γ+ intracellular staining (ICS), which measured the frequency of $CD4^+$ or $CD8^+$ T-cell secreting IFN-γ. $CD3^+$ lymphocytes were collected by simultaneously gating on $CD3^+$ events and small lymphocytes. Values higher than 0.1 % were considered positive. A positive value of 0.19 (CD8+) was obtained for monkey RI504 for pool J. No detectable anti-rhHER2 antibodies titres (> 200) were detected.

In summary, i.m. injection of plasmid DNA expressing rhesus HER2/neu was effective in breaking tolerance and inducing a detectable cell-mediated immune response against rhHER2/neu in rhesus monkeys

EXAMPLE 6

Immunization of Rhesus Macaques with Rhesus Homologs of Human Tumor-Associated Antigens

A second series of immunization studies were performed at the Biomedical Primate Research Centre (BPRC), Rijswijk (The Netherlands) in order to assess the efficiency of immunization of Rhesus macaques (*macaca mulatta*) with rhesus homologues of the human tumor antigens HER2/neu, Ep-CAM and CEA, which are all expressed in colorectal carcinomas. Protocols were designed to evaluate both B and T cell responses to these tumor antigens in combination.

In this study, a second group of 4 rhesus monkeys (2 males and 2 females) were immunized with a mixture of three plasmid DNA vectors expressing the rhesus homologues of human tumor antigens Ep-CAM (pV1J-rhHER2), CEA (pV1J-rhCEA), and HER2/neu (pV1J-rhEpCAM).

Animals were primed by i.m. injection of plasmid DNA at weeks 0, 4, 8, 12, and 16, followed by electrostimulation. The DNA injection consisted of a 1 ml solution (split over 2 sites with 0.5 ml/site) containing 6 mg plasmid DNA (2 mg for each of the three TAAs) for animals weighing 2-5 kilos. Animals were injected under anesthesia (mixture of ketamine/xylazine).

For electrostimulation, 2 trains of 100 square bipolar pulses (1 sec each), were delivered every other second for a total treatment time of 3 sec. The pulse length was 2

msec/phase with a pulse frequency and amplitude of 100 Hz and 100 mA (constant current mode), respectively.

The same group of animals was boosted by injection of a mixture of three Ad5-expressing rhesus HER2/neu (Ad5-rhHER2), rhesus CEA (Ad5-rhCEA) and rhesus EpCAM (Ad5-rhEpCAM). A total amount of 3x10exp11 viral particles (vp), were injected i.m. at weeks 23 and 27 (1x10exp11 vp for each of the three viruses).

A further boosting was carried out by i.m. injection of a mixture of three Ad24-expressing rhesus HER2/neu (Ad24-rhHER2), rhesus CEA (Ad24-rhCEA) and rhesus EpCAM (Ad24-rhEpCAM). A total amount of 3x10exp11 viral particles (vp), were injected i.m. at weeks 36 and 40 (1x10exp11 vp for each of the three viruses).

To measure the immune response to HER2/neu using the above immunization protocol, blood samples were collected every four weeks for a total duration of one year. The cell mediated immune response was measured by IFN-γ+ ELISPOT and intracellular staining, whereas the humoral response was measured by ELISA.

Monkeys RI449 and RI519 showed a detectable HER2-specific cell-mediated response, as measured by IFN-γ ELISPOT analysis. A similar analysis did not detect any response against rhCEA and rhEpCAM. No detectable anti-rheHER2 antibodies titres (> 200) were detected.

In a third study, 4 rhesus monkeys were immunized with a mixture of Ad5-rhHER2, Ad5-rhCEA and Ad5-rhEpCAM by i.m. injection of Ad5 derivatives at weeks 0, 2 and 4. A 1 ml solution (split over 2 sites with 0.5 ml/site) containing 3x10exp11 vp (10exp11 for each of the three Ad5 virus) was administered to animals weighing 2-5 kilos, under anesthesia (mixture of ketamine/xylazine).

The same group of animals was boosted at weeks 24, 26 and 28 by i.m. injection of a mixture of Ad24-rhHER2, Ad24-rhCEA and Ad24-rhEpCAM (a total amount of 3x10exp11 vp, 10exp11 vp for each of the three viruses). The cell mediated response was measured by IFNγ ELISPOT assay. For monkeys RI514 and RI496, reactivity was typed by IFN-γ+ intracellular staining. Three out of four monkeys showed a detectable response. In addition, anti-rhHER2 antibody titres ranging from 200 to 500 were detected in the three monkeys where a cell-mediated response was measured.

This immunization protocol was also effective in breaking tolerance and inducing anti-rhCEA cell-mediated and humoral immune responses. Monkey RI514 showed a measurable cell mediated response by IFN-γ ELISPOT analysis. Intracellular staining confirmed this response. The same monkey also showed anti-rhCEA antibodies titres ranging from 500 to 1000. By contrast, a similar analysis did not detect any response against rhEpCAM.

In summary, the immunization protocol discussed above was effective in inducing a specific immune response against rhHER2/neu and rhCEA in rhesus monkeys.